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14. ABSTRACT
The project was to determine how the tumor suppressor ELL-associated factor 2 (EAF2) interacts with Fork-head box protein A1 (FOXA1) and the effects of that interaction on prostate cell transcription, growth, and survival. Specific aim 1 is to determine how FOXA1 interacts with EAF2 and the specific aim 2 is to determine the effect of EAF2 on FOXA1-mediated transcription and on FOXA1-mediated cell growth and survival. Work on specific aim 1 revealed that over-expressed FOXA1 binds to EAF2 and FOXA1 does not affect EAF2 stability in the presence of cycloheximide. Work on specific aim 2 revealed that EAF2 alleviates FOXA1-mediated repression of androgen receptor transactivation. Future work will determine which amino acid stretches are required for binding, test if EAF2 can alleviate FOXA1-mediated repression of transcription in the absence of EAF2 binding to FOXA1, and investigate if EAF2 reduces FOXA1-mediated cell growth and survival.

15. SUBJECT TERMS-
prostate, prostate cancer, EAF2, tumor suppressor, protein regulation, FOXA1, androgen, receptor, PSA-expression

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Table of Contents

Introduction

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer death in men in the US (*1*). Prostate cancer results from the accumulation of mutations that affect regulatory pathways controlling cell growth, differentiation, and death. ELL-associated factor 2, also known as androgen up-regulated 19, (EAF2/U19) is a putative transcription factor and was discovered to be a protein up-regulated by the androgen receptor in the normal prostate. Over-expression of EAF2 induces apoptosis in prostate cancer cell lines while EAF2 is down-regulated in the majority of human prostate cancer specimens (*2*). Furthermore, loss of EAF2 in mice results in prostatic intraepithelial neoplasia, the putative precursor of prostate cancer (*3*).

One important question is which genes EAF2 interacts with and the effect of that interaction on the prostate. An RNAi screen was performed in the *C. elegans* model to find genes that can enhance the reduced fertility phenotype caused by loss of *eaf-1*, the *C. elegans* ortholog of EAF2 *(4)*. The screen found that knockdown of *pha-4* resulted in reduced fertility in wild-type worms, but knockdown of *pha-4* in *eaf-1* knockout worms resulted in sterility as can be seen in Figure 1. The mammalian ortholog of *pha-4* is FOXA1 (*5*). FOXA1 is a pioneer factor for the androgen receptor (*6*); FOXA1 is required in the development of prostate epithelial cells (*7*). Furthermore, upregulation of FOXA1 in prostate cancer patients is associated with a poor prognosis (*8*). We hypothesize that EAF2 associates with FOXA1 to modulate androgen receptor transactivation. This research will give a better understanding of the role of EAF2 in prostate development and prostate cancer.

The two revised and approved specific aims for the project are as follows: Specific Aim 1 will determine how FOXA1 interacts with EAF2 and Specific Aim 2 will determine the effect of EAF2 on FOXA1-mediated transcription and on FOXA1-mediated cell growth and survival. For specific aim 1, 14 months were allotted: 4 months to verify if EAF2 and FOXA1 bind exogenously and determine if they bind endogenously; 5 months to determine which amino acid regions on EAF2 and FOXA1 are required for binding; and 5 months to determine if EAF2 affects FOXA1 protein stability and if FOXA1 affects EAF2 protein stability by cycloheximide assay and by pulse-chase assay. For specific aim 2, 10 months were allotted: 2 months to determine if EAF2 affects FOXA1-mediated transcription by luciferase assay; 3 months to determine if EAF2 alters the binding of FOXA1 to DNA; 5 months to determine if EAF2 affects FOXA1-mediated cell growth and survival; and 6 months (performed concurrently with the other experiments) to determine if the physical interaction of FOXA1 and EAF2 was required for EAF2 to affect FOXA1 function.

Results

In the previous report, FOXA1 was identified by an RNAi screen as potentially interacting with EAF2. The next set of experiments tested if FOXA1 and EAF2 proteins associate in human cells. There are now four separate replications that show over-expressed EAF2 can immunoprecipitate with over-expressed FOXA1. Figure 2 shows a representative immunoprecipitation. The next two goals are to determine if FOXA1 and EAF2 bind endogenously and which amino-acid stretches are required for binding.

At the request of the primary investigator's thesis committee, the next experiment completed was a cycloheximide assay. In the previous report, it was observed that FOXA1 affects EAF2 protein levels and

therefore an assay was performed to determine if FOXA1 affects EAF2 protein stability or if EAF2 affects FOXA1 protein stability in the presence of cycloheximide, which inhibits protein translation. These experiments demonstrated that EAF2 protein levels were reduced by FOXA1, but FOXA1 did not affect EAF2 protein stability (Figure 3A). EAF2 did not affect FOXA1 protein stability (Figure 3B). However, FOXA1 protein levels increased when EAF2 was knocked out or knocked down (Figure 4). Also, PSA protein levels were reduced when EAF2 was knocked-down.

Based on the observation that loss of EAF2 affected PSA protein levels and a previous report that FOXA1 negatively regulates androgen receptor transactivation thus reducing PSA protein levels (*9*), the next experiment performed was a luciferase assay to test if EAF2 inhibits FOXA1-mediated repression of the PSA-promoter. As can be observed in Figure 5, EAF2 did relieve FOXA1-mediated repression of the PSA-promoter. This result suggests that EAF2 and FOXA1 interact to modulate the transcription of genes by the androgen receptor.

From these results, the primary investigator intends to submit a manuscript before April, 2014. This manuscript is currently titled: EAF2 Associates with FOXA1 to Alleviate FOXA1-Mediated Repression of Androgen Receptor Transactivation.

Below is an outline of the form the manuscript will take.

I. Figure 1: Knockdown of pha-4 in eaf-1 knockout *C.elegans* results in sterility.
   A. Image of wild-type + control RNAi worms, *eaf-1*KO + control RNAi worms, WT + *pha-4* RNAi worm, and *eaf-1*KO + *pha-4* RNAi worms showing improper egg development in the single and double mutants.
      a. This experiment will be performed over the next year. It should take 2-3 weeks to complete.
   B. Graph of *C.elegans* offspring number showing that *eaf-1*KO alone or *pha-4* RNAi alone causes reduced fertility, but treating *eaf-1*KO *C. elegans* with *pha-4* RNAi resulted in sterility.
      a. This experiment has been performed; please see figure 1.

II. Figure 2: FOXA1 associates with EAF2 in human prostate cells.
   A. Exogenous Co-IP.
      a. This experiment has been performed with PC3 cells; please see figure 2.
   B. Endogenous Co-IP.
      a. This experiment will be performed over the next year in LNCaP cells. It should take two months to complete.

III. Figure 3: "Binding of FOXA1 to EAF2 in human cells is mediated by specific segments of EAF2 and FOXA1."
   A. Co-IP of EAF2 fragments and FOXA1 testing for fragments that do not bind FOXA1 to determine portions of EAF2 required for association.
      a. This experiment will be performed over the next year in PC3 cells. It should take 2.5 months to complete.
   B. Co-IP of FOXA1 fragments and EAF2 testing for fragments that do not bind EAF2 to determine portions of FOXA1 required for association.
      a. This experiment will be performed over the next year in PC3 cells. It should take 2.5 months to complete.

IV. Figure 4: EAF2 alleviates FOXA1-mediated PSA-promoter repression in human prostate cells
   A. FOXA1 dosage luciferase assay.

a. This experiment has been performed using C4-2 cells; please see figure 5A.

B. EAF2 + FOXA1 luciferase assay.

a. This experiment has been performed using C4-2 cells; please see figure 5B.

V. Figure 5: "Absence of EAF2 binding to FOXA1 prevents alleviation of FOXA1-mediated promoter transcription."

A. EAF2 WT/deletion mutants + FOXA1 luciferase assay.

a. This experiment will be performed over the next year in LNCaP cells. It should take 2 months to complete.

B. FOXA1WT/deletion mutants + EAF2 luciferase assay.

a. This experiment will be performed over the next year in LNCaP cells. It should take 2 months to complete.

VI. Figure 6: "Over-expression of EAF2 counteracts FOXA1-mediated cell growth"

A. Western blots measuring cleaved Caspase 3 and PARP levels in presence of FOXA1, in EAF2, and EAF2 FOXA1. Should show increase in EAF2 alone, decrease in FOXA1 alone, intermediary in FOXA1+EAF2

a. This experiment will be performed over the next year in LNCaP cells. It should take 2 months to complete.

B. BrdU staining showing increased proliferation in presence of FOXA1, decreased in EAF2 and intermediary in EAF2+FOXA1 cells

a. This experiment will be performed over the next year in LNCaP cells. It should take 3 months to complete.

VII. Supplementary Figure 1: EAF2 is similar to *eaf-1* and FOXA1 is similar to *pha-4*.

A. Boxshade of eaf-1 and EAF2.

a. This experiment has been performed; please see figure 6A.

B. Boxshade of pha-4 and FOXA1.

a. This experiment has been performed; please see figure 6B.

VIII. Supplementary Figure 2: FOXA1 reduces EAF2 protein levels and EAF2 reduces FOXA1 protein levels.

A. Western blot showing EAF2 and FOXA1 protein levels in prostates of WT and $EAF2^{-/-}$ mice.

a. This experiment has been performed; please see figure 4A.

B. Quantification of part A western blot data using imageJ

a. This experiment has been performed; please see figure 4B.

C. Western blot showing EAF2, FOXA1, and PSA protein levels in control and EAF2 knock-down LNCaPs

a. This experiment has been performed in LNCaP cells; please see figure 4C.

D. Quantification of part C western blot data.

a. This experiment has been performed; please see figure 4D.

IX. Supplementary Figure 3: FOXA1 and EAF2 do not affect each other's protein stability in the absence of protein translation.

A. FOXA1 does not affect EAF2 protein stability

a. This experiment has been performed in LNCaP cells; please see figure 3A.

B. EAF2 does not affect FOXA1 protein stability.

a. This experiment has been performed in LNCaP cells; please see figure 3B.

After this manuscript is submitted for publication, the primary investigator will then submit a thesis based on the data mentioned in the outline and receive their Ph.D.

Key Research Accomplishments

- FOXA1 and EAF2 bind when both are transfected
- FOXA1 alters EAF2 protein levels, but not protein stability
- FOXA1 protein levels are elevated when EAF2 is knocked-down or knocked out, and PSA protein levels decrease when EAF2 is knocked-out
- EAF2 alleviates FOXA1-mediated repression of androgen receptor-mediated transcription of the PSA-promoter.

Reportable Outcomes

- Presented a poster at the Great Lakes Nuclear Receptor Conference

Conclusion

The current data supports the hypothesis that EAF2 associates with FOXA1 to modulate androgen receptor transactivation. Progress has been made on both specific aims. For specific aim 1, co-IPs performed using transfected FOXA1 and EAF2 showed FOXA1 and EAF2 bind. Also, cycloheximide assays showed that while FOXA1 affects EAF2 protein levels, EAF2 protein stability is not affected. However, western blots using EAF2 KO mouse prostates or EAF2 knockdown LNCaP cells showed FOXA1 protein levels increase when EAF2 protein levels are reduced. For specific aim 2, luciferase assays performed using PSA-luciferase showed that EAF2 alleviates FOXA1-mediated repression of androgen receptor transactivation. The results will serve as the basis for a paper that should be submitted for publishing before March 30th. This work will create a better understanding of the protein interactions in the normal prostate that are disrupted in prostate cancer.

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Appendix A: Abstracts

2012 Great Lakes Nuclear Receptor Conference Abstract

First Name: Anne

Last Name: Keener

Other Authors: Liquan Cai, Junkui Ai

Advisor: Zhou Wang

Abstract Title: Exploring mechanisms of EAF2 action – from RNAi screen in *C. elegans* to mammalian analysis

EAF2 (ELL-associated factor 2) (also known as androgen up-regulated 19 or U19) is a RNA polymerase II transcription elongation factor. EAF2 positively regulates Eleven-Nineteen Lysine Rich Leukemia Factor (ELL), another RNA polymerase II transcription elongation factor. EAF2 is a tumor suppressor and it is up-regulated by the androgen receptor in the normal prostate. Over-expression of EAF2 in cancer cell lines causes apoptosis and when EAF2 is knocked down in mice, mice develop leukemia, liver carcinoma, lung adenocarcinoma, and prostate carcinoma. The *C. elegans* ortholog to EAF2 is eaf-1. When eaf-1 is knocked out in *C. elegans*, the resulting worms have reduced fertility, cuticle malformation, and have the phenotype known as "dumpy," i.e. are smaller than WT worms. The eaf-1KO *C. elegans* worms were used to screen for enhancers, genes that make a known phenotype worse. Knockdown of the gene pha-4 was found to render the eaf-1KO worms sterile. The mammalian ortholog to pha-4 is FOXA1 (Forkhead Box A1), a pioneer factor for the androgen receptor. FOXA1 is required for normal differentiation of prostatic epithelial cells. We hypothesized that FOXA1 is essential for the regulation of EAF2. Co-immunoprecipitations (Co-IPs) were performed using tagged FOXA1 and EAF2 to test if EAF2 and FOXA1 bind. There is preliminary evidence that FOXA1 is in a complex with EAF2. Cycloheximide assays were also performed to test if FOXA1 affects EAF2 protein levels. EAF2 levels are reduced in the presence of FOXA1, but FOXA1 does not affect the degradation rate of EAF2. More work is needed to verify these results.

Supporting Data

Figure 1

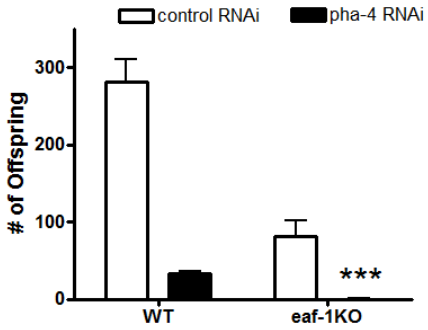

Figure 1: **Loss of *pha-4* in *eaf-1* KO *C. elegans* results in sterility**
Knockdown of *pha-4* in wild-type cells resulted in a significant reduction in number of offspring compared to wild-type treated with control RNAi. Knockout of *eaf-1* (*eaf-1* KO) resulted in a significant reduction in number of offspring compared to wild-type. Knockdown of *pha-4* in *eaf-1* KO *C. elegans* resulted in sterility.
***=p<0.001

Figure 2

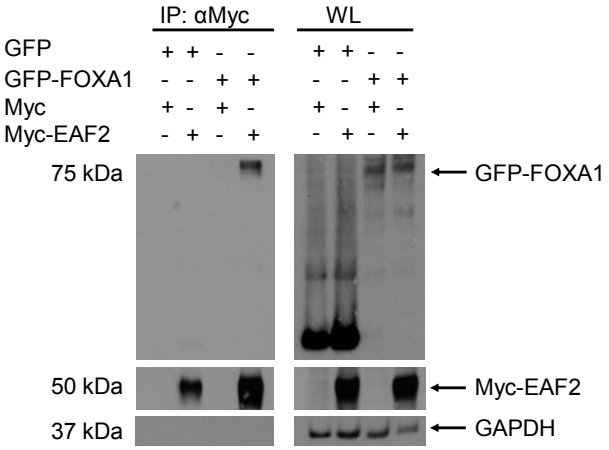

Figure 2: **FOXA1 and EAF2 associate** Representative Co-IP of transfected FOXA1 and EAF2. Four experiments were performed in PC3 cells.

Figure 3

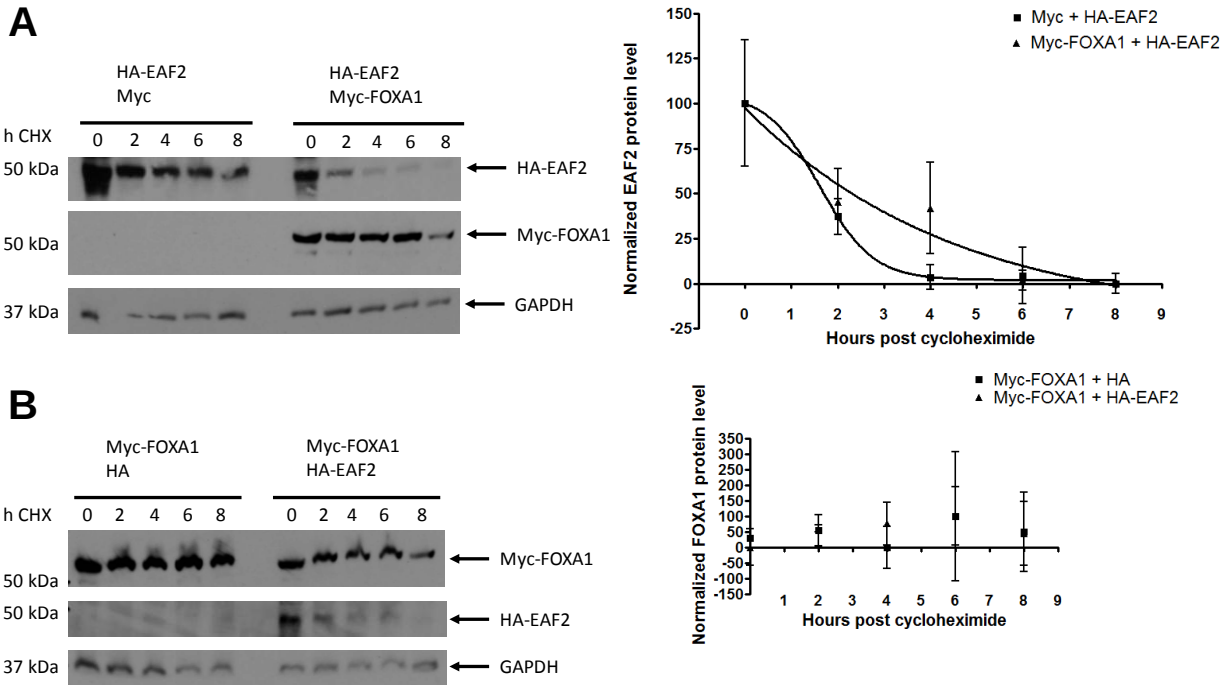

Figure 3: **FOXA1 does not affect EAF2 protein stability and EAF2 does not affect FOXA1 protein stability** A) FOXA1 does not affect EAF2 protein stability. Representative blot of results. Three were performed in LNCaP cells. EAF2 protein levels were normalized to GAPDH. B) EAF2 does not affect FOXA1 protein stability. Representative blot of results. Three were performed in LNCaP cells. FOXA1 protein levels were normalized to GAPDH.

Figure 4

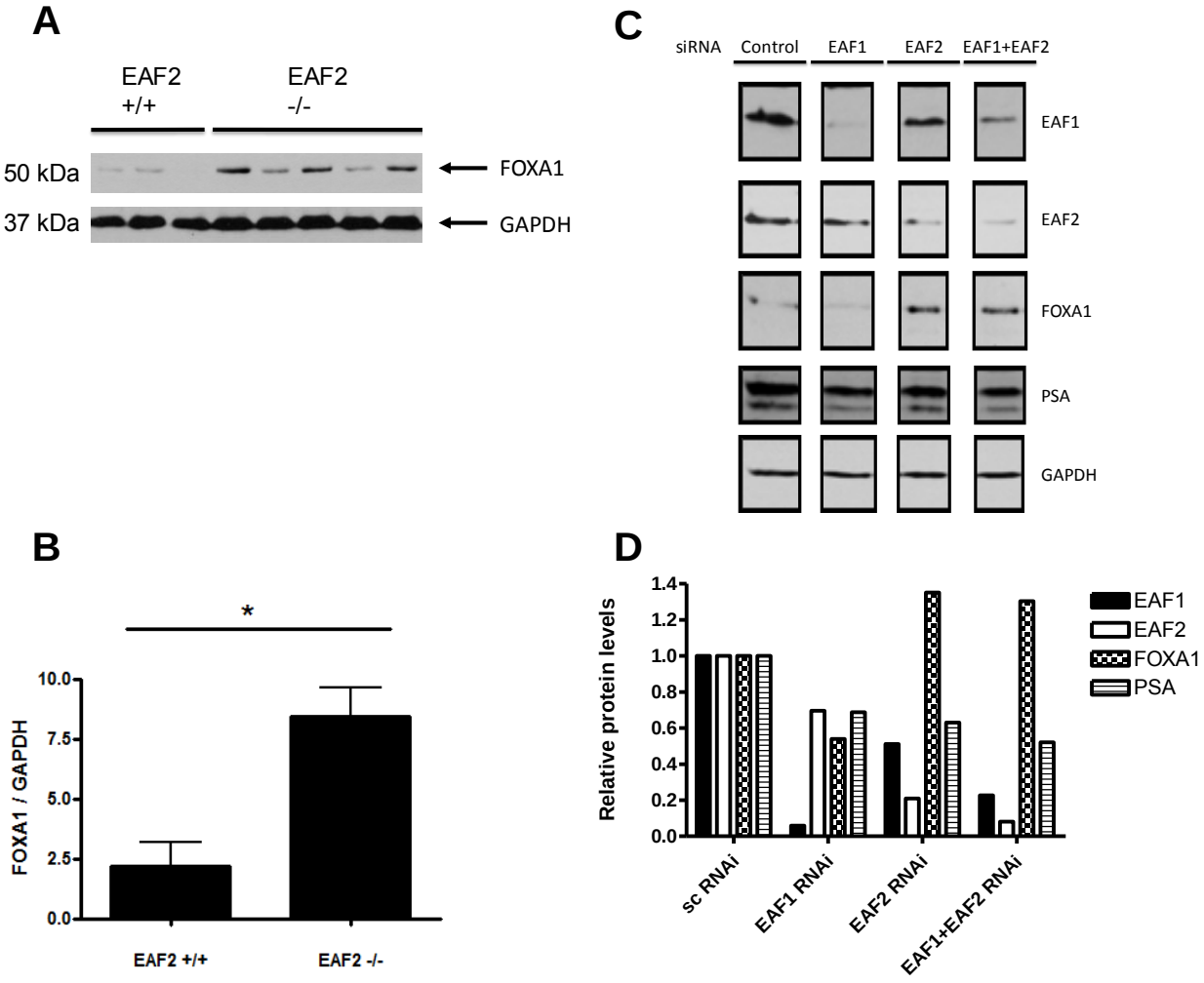

Figure 4: **FOXA1 decreases EAF2 protein level and EAF2 reduces FOXA1 protein level** A) FOXA1 protein levels rise in the prostates of EAF2 knockout mice compared to wild-type mice. B) Plot of relative protein levels normalized to GAPDH. C) FOXA1 protein levels rise when EAF2 is knocked-down in LNCaP cells but not when EAF1 is knocked-down. PSA levels decrease when EAF2 is knocked-down. D) Plot of protein levels from 4C normalized to GAPDH.

Figure 5

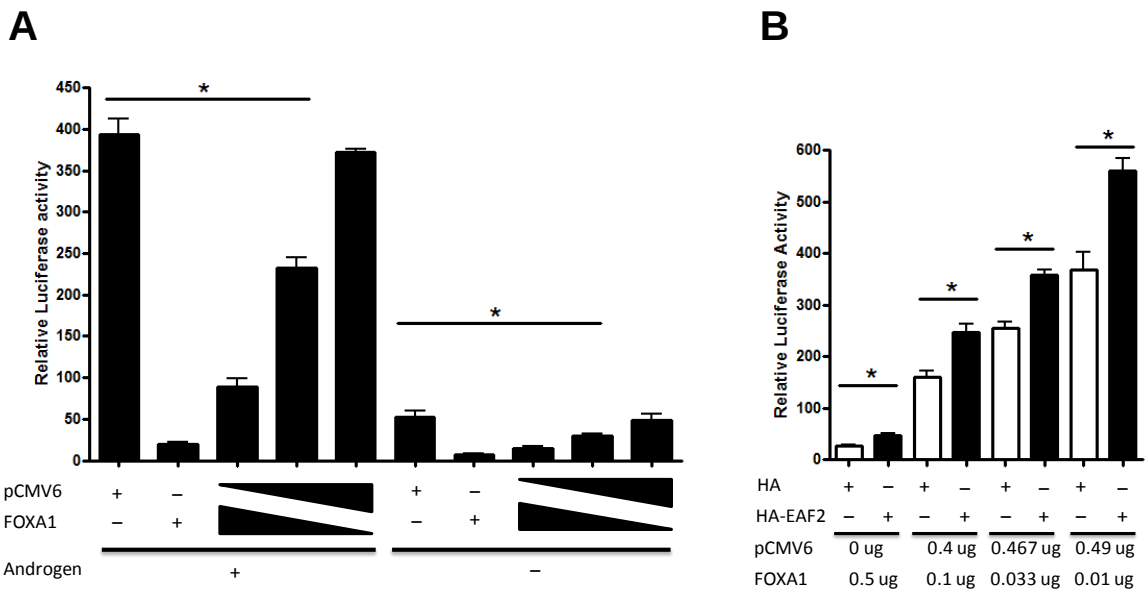

Figure 5: **EAF2 alleviates FOXA1-mediated PSA-promoter repression** A) Activity of PSA-luciferase in the presence of FOXA1. Samples where performed with or without added androgen (1nM R1881). All samples were normalized to renilla. B) Activity of PSA-luciferase in the presence of FOXA1 when EAF2 is over-expressed. All samples performed in the presence of 1nM R1881. All samples were normalized to renilla. *=p<0.05 All experiments were performed in C4-2 cells.

Figure 6

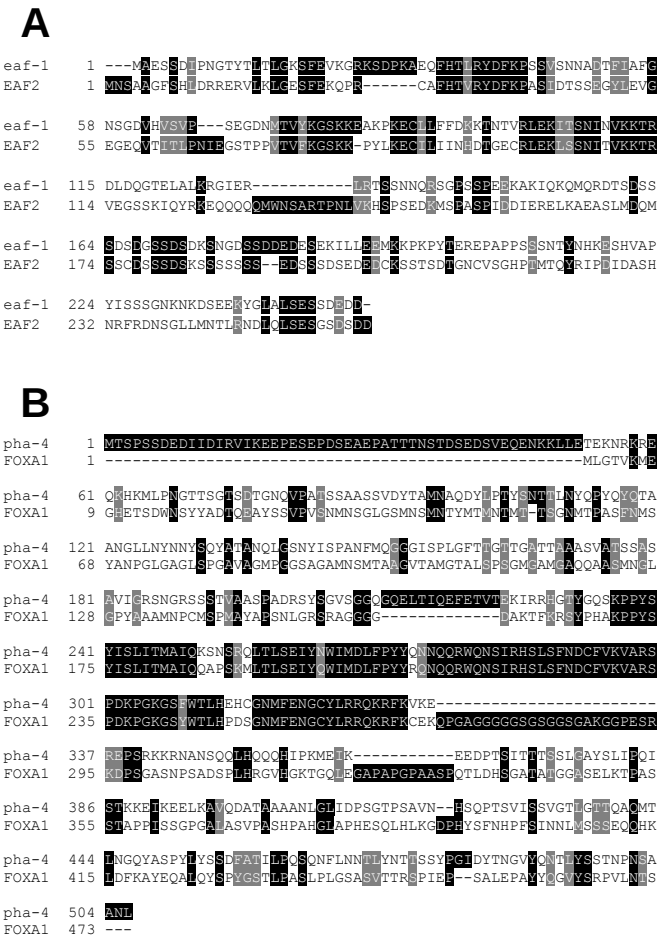

Figure 6: **FOXA1 is the human ortholog of pha-4 and EAF2 is the human ortholog of eaf-1** A)The human ortholog of eaf-1 is EAF2. EAF2 has 58.3% similarity with eaf-1. B) The human ortholog of pha-4 is FOXA1. There is 61.9% similarity between FOXA1 and pha-4.